Characterization of the Aggregation-Prevention Activity of p97/Valosin-Containing Protein†

Changcheng Song,*,‡,§ Qing Wang,‡ and Chou-Chi H. Li‡,§

Laboratory of Cancer Prevention and Basic Research Program, SAIC-Frederick, Inc., NCI-Frederick, Frederick, Maryland 21702

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ABSTRACT: The 97 kDa valosin-containing protein (VCP) belongs to a highly conserved AAA (*A*TPases *a*ssociated with a variety of *a*ctivities) family and contains two ATPase domains, D1 and D2. VCP participates in numerous cellular activities, such as membrane fusion, postmitotic Golgi reassembly, endoplasmic reticulum-associated degradation, ubiquitin−proteasome-mediated proteolysis, and many others. In performing these activities, VCP presumably acts as a molecular chaperone that prevents protein aggregation and modifies protein conformation. In this study, we characterized the aggregation-prevention activity of VCP and identified the structural requirement for this activity. We used multiple methods to treat aggregation-prone luciferase (Luc) and showed that VCP prevents the aggregation of Luc in vitro. These results are in agreement; in vivo RNA interference analyses showed that a reduction of VCP level results in more aggregation of Luc in cells. Structural and functional analyses further demonstrated that the D1 domain of VCP is sufficient to mediate the aggregation-prevention activity, which does not require ATP binding, ATP hydrolysis, or a hexameric structure of VCP. Together, these results indicate that (1) VCP prevents protein aggregation in vitro and in vivo, (2) this aggregation-prevention activity is mediated mainly through the D1 domain of VCP, and (3) this activity does not require ATPase activity or a hexameric structure of VCP.

VCP is one of the most conserved genes across the evolutionary scale. It is also known as VAT in archaeal bacteria, CDC48 in yeast, TER94 in *Drosophila*, p97 in *Xenopus*, and p97/VCP in mammals (*1*−*5*). It is an essential gene and codes for an abundant 97 kDa ATPase that belongs to the ancient AAA (ATPases associated with a variety of activities) family (*6*; reviewed in refs *7*−*12*). As other members of the family, VCP is involved in a wide variety of cellular activities, including membrane fusion, Golgi reassembly after mitotic division, endoplasmic reticulum-(ER-) associated degradation (ERAD), ubiquitin−proteasome-mediated proteolysis, and others (reviewed in ref *13*). The participation of VCP in these seemingly unrelated functions relies on its conserved chaperone activity. We propose that VCP, like many molecular chaperones, readily binds non-native proteins and modifies their conformation so that these client proteins are properly prepared to carry out the next activity. For example, in mediating membrane fusion, VCP, along with the cofactors, catalyzes the disassembly of the SNARE (soluble *N*SF *a*ttachment protein *re*ceptors) complex following the fusion event, thus allowing the individual SNARE proteins to be recycled for further rounds of fusion (*14*). In ubiquitin−proteasome-mediated degradation, VCP likely acts as a polyubiquitin-specific segregase that binds to the ubiquitinated substrate protein, which is usually associated with, and protected by, other proteins, and then separates this substrate from the complex and targets it to the proteasome for degradation (*15*).

In recent years, we and other groups conducted a series of studies on VCP at a molecular level to characterize its biological functions and the structural requirement for each of these functions. Structurally, the VCP molecule consists of three clearly defined domains: the N domain (residues 1−187) and two conserved ATPase domains, D1 (residues 209−460) and D2 (residues 481−763) (*16*). We and others previously showed that the N domain binds to polyubiquitinated proteins (*17*−*19*) and cofactors of VCP (*20*). Structural studies have shown that six neighboring ND1 (residues 1−481) domains form a stable hexameric ring-shaped structure, in which D1 is the major mediator for hexamerization (*21*, *22*). We also demonstrated that the neighboring D2 domains, which also form a ring-shaped structure, are the major mediators for the ATPase activity (*23*). Furthermore, the D1 ring remains relatively stable, and the D2 ring undergoes major conformational changes during ATP hydrolysis reactions (*21*).

Previous studies suggest that VCP is involved in many cellular activities by acting as a molecular chaperone. VCP is expected to perform activities such as binding to non-native proteins, suppressing protein aggregation, disassembling protein complexes, and perhaps even unfolding or refolding the client proteins. However, the molecular mechanisms involved in these activities are not well understood. In this study, we investigated the basic chaperone activity of VCP as an inhibition of aggregation and found that it prevents the aggregation of non-native proteins in an ATP-independent manner. With a variety of VCP mutants, we

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* Address correspondence to this author. Phone: (240) 285-8586. Fax: (240) 683-6892. E-mail: songc@ncifcrf.gov.

‡ Laboratory of Cancer Prevention, NCI-Frederick.

§ Basic Research Program, SAIC-Frederick, Inc.

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further showed that this basic chaperone activity does not require the ATPase activity or a hexameric structure of VCP and the monomeric D1 domain is sufficient to prevent protein aggregation. In addition, we used the RNA interference technique to reduce the level of VCP in the cells that stably express a fusion protein comprising firefly luciferase (Luc) and green fluorescent protein (GFP). Luc-GFP serves as a reporter for protein aggregation, in which the thermolabile Luc molecules form aggregates in response to heat shock, while GFP remains stable to produce green fluorescence (*24*). Complementary to the in vitro results, we found that the level of VCP inversely correlates with the extent of Luc-GFP aggregation under heat shock conditions. Finally, the results show that VCP associates with the non-native Luc molecules and colocalizes, with the foci containing Luc-GFP aggregates.

MATERIALS AND METHODS

Plasmids, Proteins, and Reagents. The wild-type (WT) and mutant VCP proteins were constructed and expressed as fusion proteins, each with a hexahistidine (His) peptide tagged at the C-terminus (*23*). N (residues 1−200), ND1 (residues 1−481), D2 (residues 443−806), and D1D2 (residues 208−806) have been previously reported under the names of N200, N-D1-linker, E, and delta N, respectively (*21*−*23*). Other mutants were generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). D1 (residues 208−481) was generated using ND1 as the template and oligonucleotide 5′-GAGAAATTAACCATGGAAGTAGGCTATGATG-3′ and its complementary strand as primers. D1D2 (residues 208−806) was generated using WT as the template and oligonucleotide 5′-GGTACCGGACATATGGGTGGTTGCAGGAAG-3′ and its complementary strand as primers. A1A2 (K251T/K524T) is a site-specific mutant that contains K to T mutations at the ATP-binding site in both D1 and D2 domains. ND1-R359A and ND1-R362A have been reported under the names of [1−481]-R359A and [1−481]R362A (*25*), respectively. All His-tagged recombinant proteins were expressed and purified according to the manufacturer's specifications (Qiagen Inc., Valencia, CA) and stored in the dialysis buffer (DB: 50 mM Tris-HCl, pH 8.0, 5% glycerol, 1 mM DTT). GroEL was purchased from Stressgen Biotechnology Corp., Ann Arbor, MI. Firefly Luc, *N*-ethylmaleimide (NEM), and ATP were purchased from Sigma. The reporter plasmid, pLuc-GFP, that encodes the Luc-GFP fusion protein (*24*) was kindly provided by H. Kampinga, Groningen, The Netherlands. The monoclonal anti-VCP antibody was produced by immunizing the mouse with a KLH-conjugated synthetic peptide corresponding to the C-terminal 792−806 residues of human VCP and affinity purified by protein G chrotomatography.

In Vitro Luc Aggregation Assay. To assay heat-induced aggregation of Luc, 0.15 μM Luc in buffer A [12.5 mM HEPES, pH 7.9, 2.5 mM $Mg(OAc)_2$, 25 mM KCl, 2.5 mM β-mercaptoethanol] was heated at 43 °C in the presence of different amounts of VCP. Aggregation of Luc was determined by measuring the light absorption at 320 nm in 5 min intervals using a Beckman Du640 UV−vis spectrophotometer. To assay urea-induced aggregation, the basic procedure was based upon the previously reported method (*26*). Luc (7.5 μM) was denatured in buffer B (25 mM HEPES-KOH, pH 7.6, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT) containing 8 M urea for 30 min and then diluted 50-fold with the same buffer with or without test chaperones at room temperature. Light scattering of the protein aggregates was determined by measuring the absorbance at 320 nm in 10 s intervals. For the experiments described in Figures 1d and 2d, reactions were centrifuged at 12000g for 10 min, and the pellet and supernatant fractions from each reaction were analyzed by SDS−PAGE followed by Western blot using Luc-specific antiserum (Sigma, St. Louis, MO).

Cell Culture and Transfection. Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal calf serum (BD Biosciences, Bedford, MA) at 37 °C in a humidified atmosphere with 5% CO_2. Luc-GFP (*24*) plasmid DNA was transfected into the cells using Lipofectamine 2000 (Invitrogen, Inc., Carlsbad, CA). A stable Luc-GFP-expressing cell line was established by flow cytometry sorting for GFP-positive cells and then used in RNA interference and heat shock experiments.

RNA Interference. HEK293 cells stably expressing Luc-GFP were first transfected with two shRNA plasmids using Superfect (Qiagen Inc.) according to the manufacturer's instructions. The two targeting regions of the VCP gene are 5′-CGGTTCTTCTGTTTGAGAATGGCTGTTGA-3′ and 5′-TTTAGGCCATCCATGAGGGTCAACAACTG-3′ (Open-Biosystems Inc., Huntsville, AL). For the control, cells were transfected with the empty vector of shRNA plasmid.

In Vivo Protein Aggregation Analysis. The Luc-GFP-expressing HEK293 cells transfected with shRNAs or their control vector were heat-shocked at 43 °C for 1 h or kept at 37 °C as a control. Cells were washed twice with cold PBS containing 1 mM PMSF, resuspended in 100 μL of PBS buffer, and lysed by sonication (7 × 7 s) with a Branson Sonifier 250 sonicator. The soluble and insoluble fractions of cell lysates were separated by 12000*g* centrifucation for 10 min and then analyzed by SDS−PAGE and subsequent Western blotting with antibodies against VCP, Luc, and β-actin.

Luc Activity Assay. The Luc enzyme activity was assayed with a Luc assay kit (Promega Inc., Madison, WI) following the manufacturer's protocol. In brief, 2.5×10^4 cells transfected with VCP shRNA or control vector were heat shocked at 43 °C for 1 h and then lyzed in Glo buffer. Aliquots of 2 μL of cell lysates were mixed with 50 μL of Steady-Glo Luc assay reagent at room temperature, and the enzyme activity of Luc was measured using the GENios multifunction reader (Tecan Group Ltd., San Jose, CA).

Immunofluorescence Analysis. HEK293 cells expressing Luc-GFP were grown and processed in Lab-Tek chambers (Nalgene Nunc Int. Corp., Rochester, NY). For immunofluorescence analyses, the cells were washed twice with cold PBS, fixed with ice-cold methanol, washed three times with cold PBS, and incubated with a blocking buffer (TBS buffer with 0.1% bovine serum albumin and 0.1% gelatin) for 30 min. The cells were then reacted with VCP C-terminus-specific antibody (*17*), followed by the TRITC-conjugated anti-mouse antibody (Sigma). The same samples were also stained with Hoechst 33342 (3 μg/mL; Sigma) to localize nuclei. Finally, cells were washed three times with cold TBS, and Luc-GFP, VCP, and nuclei were localized by imaging GFP, TRITC, and Hoechst 33342, respectively. The images were acquired using Zeiss LSM510 confocal laser-scanning microscopy.

Pull-Down Assay. Luc (100 nM) was mixed with different VCP-His variants (1 μM each) in buffer A [12.5 mM

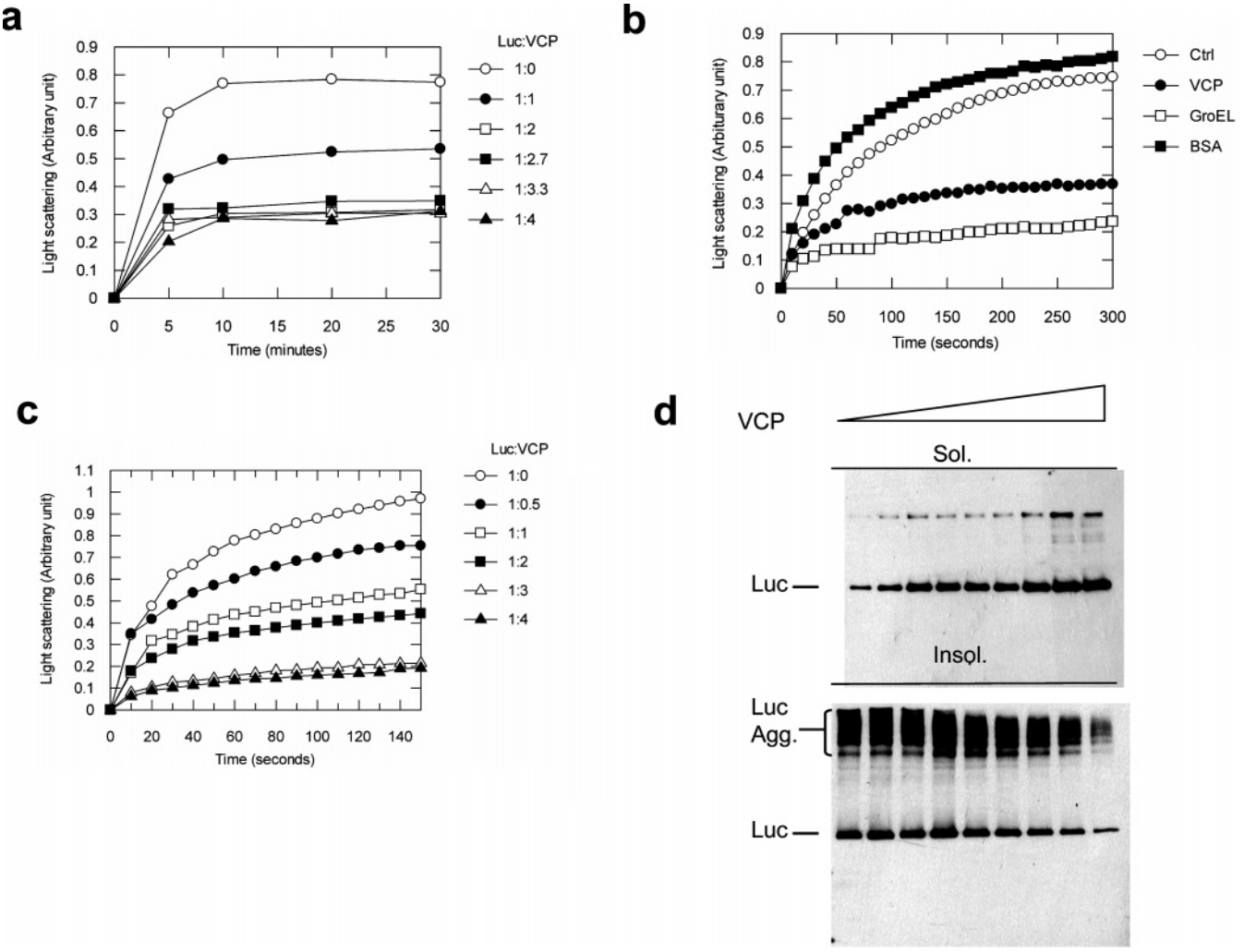

FIGURE 1: VCP prevents the aggregation of denatured Luc. (a) Luc (0.15 μM) was incubated in the designated ratio with VCP at 43 °C for 30 min. (b) Luc was treated with 8 M urea and diluted to 0.15 μM with either the control buffer (○) or buffer with 1 μM VCP (●), 1 μM GroEL (□), or 1 μM BSA (■). (c) Urea-treated Luc was diluted to 0.15 μM with a buffer containing 0, 0.08, 0.15, 0.30, 0.45, or 0.60 μM VCP. The turbidity in each reaction was measured kinetically as light absorption at 320 nm in 10 s intervals. (d) Urea-treated Luc was diluted to 100 nM with the buffer containing an increasing amount of VCP. VCP (concentration in monomers): Luc = 0, 0.5, 1, 2, 3, 4, 8, 16, and 32 in lanes from left to right. After incubation at 25 °C for 15 min, the reactions were centrifuged, and equal portions of the supernatant and pellet were analyzed by SDS−PAGE and Western blot with anti-Luc antibody.

HEPES, pH 7.9, 2.5 mM $Mg(OAc)_2$, 25 mM KCl, 2.5 mM β-mercaptoethanol] and incubated at 43 or 4 °C for 10 min. Each reaction was centrifuged at 5000*g* for 5 min, and the supernatant (which contains VCP and the stressed, non-native Luc) was reacted with Ni-NTA agarose beads to pull down VCP and the potentially associated Luc. The collected VCP-containing complex was subsequently analyzed by SDS−PAGE followed by Western blotting with anti-Luc antibody. The detection of Luc indicates a physical association between VCP and Luc.

Co-immunoprecipitation. The Luc-GFP-expressing HEK293 cells were challenged at 43 °C for 1 h and lyzed in 200 μL of cold PBS supplemented with 1 mM PMSF. Cell lysates were centrifuged at 5000*g* for 5 min at 4 °C, and the supernatant was immunoprecipitated with monoclonal anti-VCP antibody and protein G−Sepharose beads for 2 h at 4 °C. The precipitated VCP complexes were washed three times with cold PBS and subjected to SDS−PAGE and Western blot. The coprecipitated Luc was detected by Western blotting with anti-Luc antibody.

RESULTS

VCP Prevents Protein Aggregation in Vitro. To study the chaperone activity of VCP at a molecular level, we first examined the capability of VCP to prevent protein aggregation in vitro. We used Luc as a reporter for stress-induced denaturation and aggregation since it is highly sensitive to both heat and chemical stress and has been commonly used to analyze chaperone activity in vitro and in vivo. We thermally denatured Luc in the presence of different amounts of VCP and then detected the aggregation by optical absorption. As shown in Figure 1a, heat-denatured Luc rapidly formed aggregates in the absence of a chaperone (top curve), and the aggregation was efficiently suppressed in the presence of VCP (lower curves). The suppression correlates with the concentration of VCP.

In addition, we chemically denatured Luc with 8 M urea and then diluted it in the presence of various agents. As shown in Figure 1b, the denatured Luc molecules formed aggregates in the absence of chaperones (○) and in the presence of a control protein BSA (■). In contrast, the aggregation was significantly suppressed by VCP (●) and

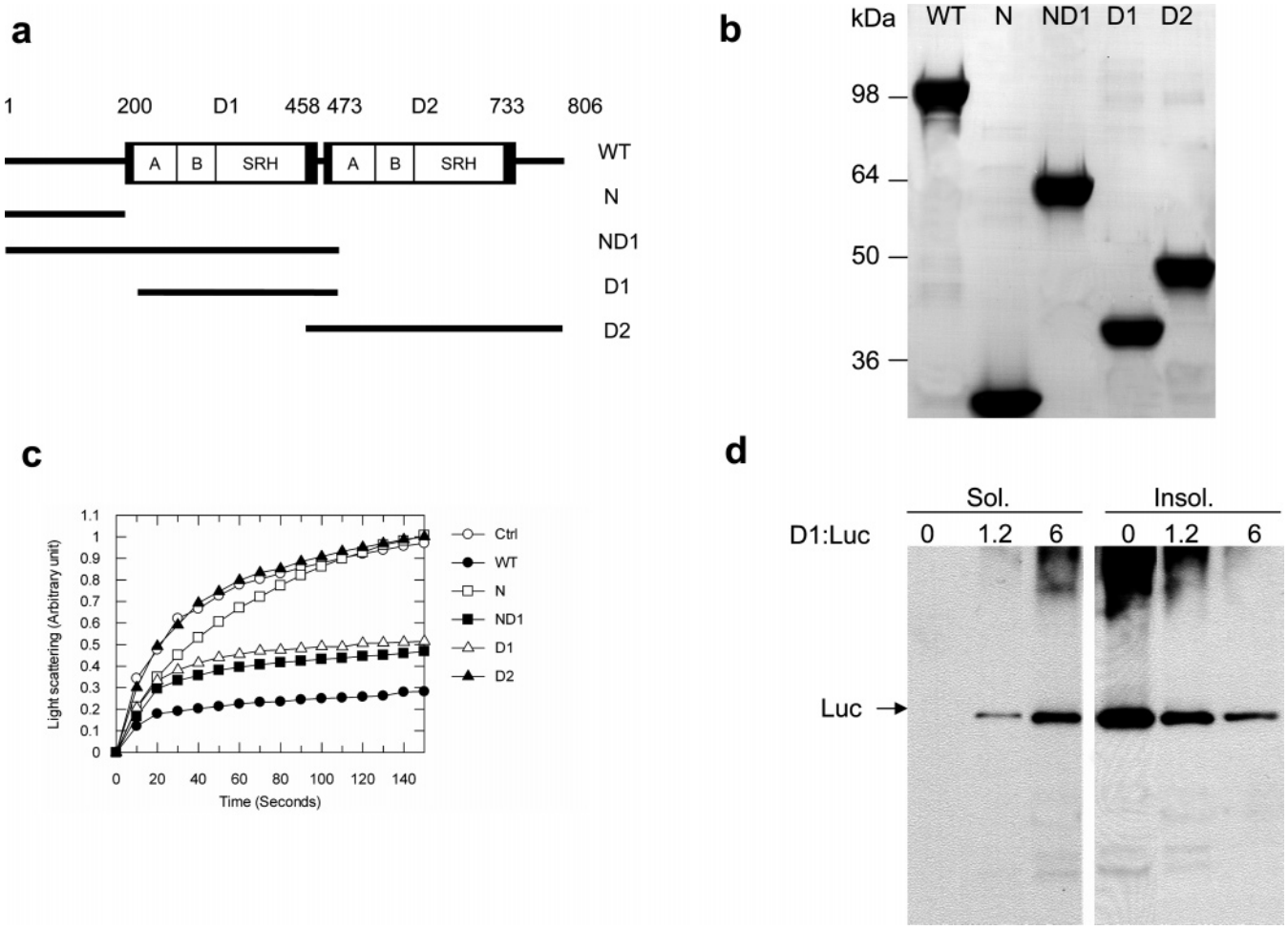

FIGURE 2: The VCP D1 domain is the major mediator for aggregation prevention. (a) Gene structures of WT VCP and the truncated mutants (N, ND1, D1, and D2) are shown. (b) The expressed WT and the mutant proteins were purified from *E. coli*, resolved by SDS−PAGE gel, and stained with Coomassie Blue. The molecular size standards are shown on the left. (c) Urea-induced Luc aggregation was performed in the presence of the designated VCP variants, and the aggregation was monitored. (d) Urea-treated Luc was diluted to 100 nM with buffer containing an increasing concentration of D1 protein (as shown in the ratio). After incubation at 25 °C for 15 min, the reactions were centrifuged, and equal portions of the supernatant or the pellet were analyzed by SDS−PAGE and Western blotting with Luc-specific antibody.

GroEL (□), a prototype molecular chaperone. Further analyses showed that this activity was VCP dosage dependent (Figure 1c). To further analyze the Luc aggregates, the reactions were centrifuged and separated into a soluble fraction containing dissolved Luc molecules or an insoluble fraction containing Luc aggregates. The separated fractions were analyzed by SDS−PAGE followed by immunoblotting. As expected, when an increasing level of VCP is present in the reaction, more Luc was detected in the soluble fraction and less in the pellet (Figure 1d). Thus, the degree of suppression in protein aggregation shows a correlation with the increase in VCP concentration.

The VCP D1 Domain Plays an Important Role in Mediating Aggregation Prevention. To determine which domain of VCP is responsible for the aggregation-prevention activity, WT and truncated mutants (Figure 2a), including N (residues 1−200), ND1 (residues 1−481), D1 (residues 208−481), and D2 (residues 443−806), were expressed, purified (Figure 2b), and used in assays for urea-induced Luc aggregation. We found that the variants containing D1, WT, ND1, and D1 prevented aggregation significantly, but N and D2, the truncations lacking the D1 domain, did not (Figure 2c). Fractionation of the reactions by centrifugation further showed that when an increasing amount of D1 was present in the reaction, more Luc was detected in the soluble fraction and less in the insoluble aggregates (Figure 2d). Therefore, D1 appears to play an important role in mediating the aggregation-prevention activity of VCP.

Hexamerization Is Not Required for VCP To Prevent Protein Aggregation. It has been shown that an oligomeric structure is critical for many biological functions of AAA family members (*10*). Our previous studies also showed that a hexameric structure of VCP is essential for its ATPase activity (*21*, *22*). To test whether the hexameric form is also required for aggregation-prevention activity, we first analyzed the oligomeric state of all the mutants presented in Figure 2 by native gel electrophoresis (Figure 3). Consistent with previous results, WT and ND1 formed hexamers (lanes 1 and 3), but N and D2 did not (lanes 2 and 5). Surprisingly, in contrast to ND1, which was predominantly hexameric (lane 3), D1 did not oligomerize to form a stable hexamer (lane 4), Since we observed that D1 sufficiently prevents protein aggregation (Figure 2), this result suggests that the aggregation-prevention activity does not require a hexameric structure of VCP. To further substantiate this finding, we expressed and purified ND1 and ND1-derived mutants (Figure 4a,b) and compared their aggregation-prevention activities. ND1 proteins, as expected, formed hexamers, but

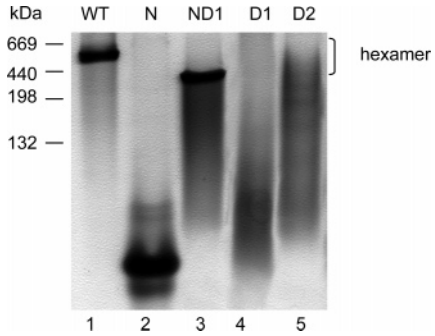

FIGURE 3: VCP variants containing the D1 domain are not detected in a hexameric form. The purified His-tagged VCP proteins (5 μg each), including WT, N, ND1, D1, and D2, were resolved on a native polyacrylamide gel and stained with Coomassie Blue. The molecular size standards are shown on the left. The hexameric form is indicated.

the SRH site-specific mutants, ND1-R359A and ND1-R362A, were predominantly monomers (Figure 4c). Consistent with the results obtained from D1 variants (Figure 2), all of these ND1 variants, without hexamers or not, significantly suppressed the aggregation of urea-treated Luc (Figure 4d). These results strongly support the conclusion that a hexameric structure is not required for the aggregation-prevention activity of VCP.

Aggregation-Prevention Activity Does Not Require ATP. Since VCP undergoes major conformational changes upon ATP binding (*21*, *27*), we further tested whether ATP binding affects the aggregation-prevention activity of VCP. We treated Luc with urea and then diluted it with or without VCP. In each set of reactions, aggregation was measured after the treated Luc was incubated with a buffer containing 0, 1, or 3 mM ATP. As shown in Figure 5a, the presence of VCP significantly suppresses the aggregation (lower curves) compared to the set without VCP (upper curves). Moreover, the ATP concentration does not seem to change the extent of aggregation in either set of reactions, suggesting that the activity does not depend on ATP. Furthermore, we examined whether this aggregation-prevention activity requires the ATPase enzyme activity of VCP. We found that the alkylating agent NEM, which completely inhibits the ATPase activity of VCP (*2*), did not change the ability of VCP to suppress Luc aggregation (Figure 5b). Together, these results suggest that the aggregation-prevention activity required neither ATP binding nor ATP hydrolysis. To further confirm this observation, we used the mutant A1A2, which harbors mutations in the ATP-binding site in both D1 and D2. We found that A1A2 is still capable of suppressing the aggregation of Luc, although less efficiently than WT VCP (Figure 5c). Therefore, the aggregation-prevention activity of VCP is not significantly affected by the conformational changes induced by ATP binding or ATP hydrolysis.

The D1 Domain of VCP Binds to Denatured/Non-native Luc. On the basis of the finding that D1 is required for aggregation-prevention activity, we considered whether D1 protein actually binds to the denatured/non-native Luc. We incubated Luc with His-tagged VCP variants under denaturing condition (43 °C) and then centrifuged the reactions. The insoluble fraction should contain the aggregates of denatured Luc, and the soluble fraction should contain the molecular chaperone, VCP, and its associated target, the denatured/non-native Luc. We added Ni-conjugated beads to the soluble fraction to isolate the complex of His-tagged VCP and its associated target proteins. This VCP-containing complex was then analyzed by SDS−PAGE followed by immunoblotting to detect Luc. As shown in Figure 6a, Luc was only

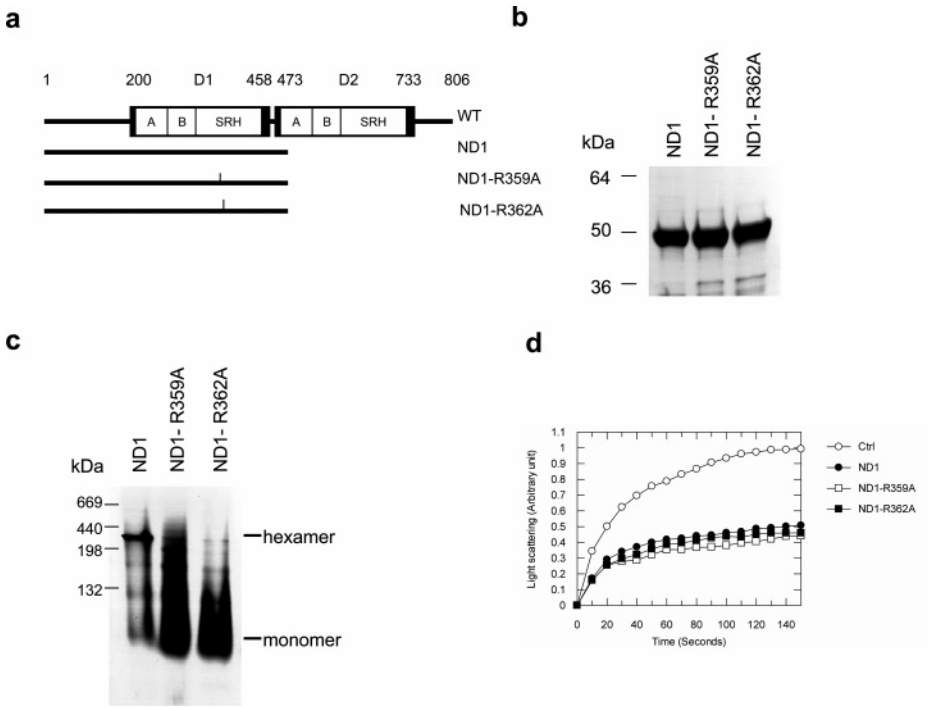

FIGURE 4: Both hexameric and nonhexameric ND1 (residues 1−481) variants can suppress protein aggregation. (a) Gene structure of the wild-type ND1 and its site-specific mutants, ND1-R359A and ND1-R362A, are depicted. (b) The proteins of ND1 variants were expressed, purified, resolved by SDS−PAGE, and stained with Coomassie Blue. (c) The purified proteins of ND1 variants were resolved by native gel electrophoresis and stained with Coomassie Blue. The molecular size standards are shown on the left, and the hexamer and monomer are indicated. (d) Urea-treated Luc was diluted to 0.15 μM in a buffer containing no VCP (○), ND1 (●), ND1-R359A (□), or ND1-R362A (■) (0.45 μM each). Light scattering was measured kinetically as light absorption at 320 nm in 10 s intervals.

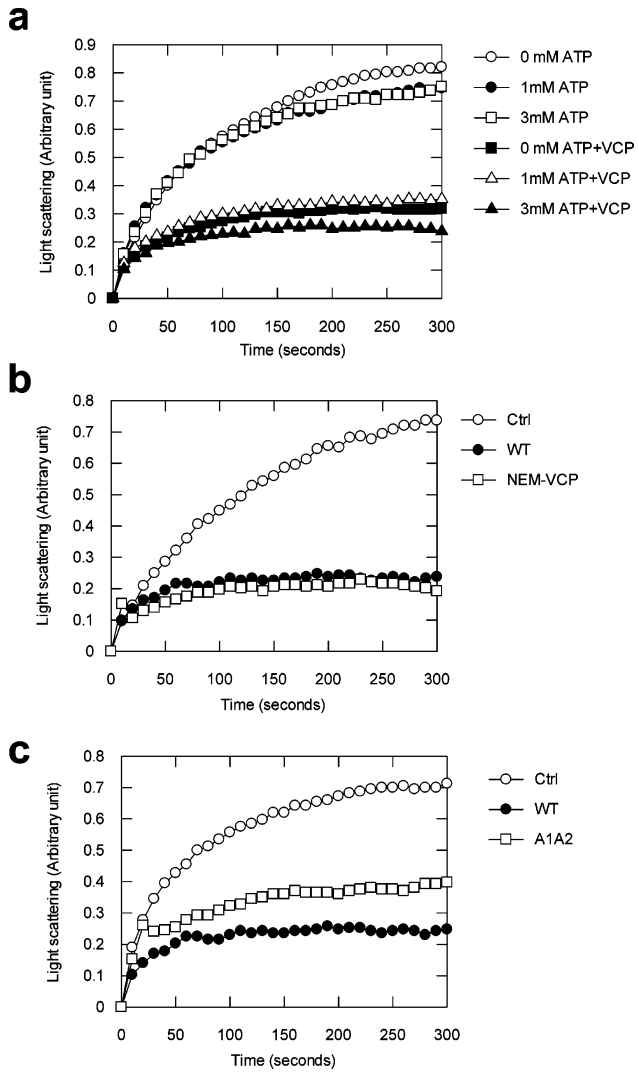

FIGURE 5: Aggregation-prevention activity of VCP is ATP-independent. Urea-treated Luc was diluted to 0.15 μM with a buffer containing (a) 0, 1, or 3 mM ATP with (lower curves) or without VCP (upper curves); (b) no VCP (○), wild-type VCP (●), or NEM-inactivated VCP (□); (c) no VCP (○), WT VCP (●), or the A1A2 mutant (□). Reactions described in (b) and (c) contain 1 mM ATP, and the concentrations for all tested VCP variants are 1 μM. Light scattering was measured kinetically as light absorption at 320 nm in 10 s intervals.

coprecipitated with those VCP variants possessing the D1 domain, including the WT, ND1, D1, ND1-R359A, and ND1-R362A proteins. In contrast, N and D2, in which D1 is absent, did not bind to the non-native Luc. This result strongly suggests that D1 was required for binding to the non-native proteins. To further examine whether this binding was affected by the presence or absence of ATP, we performed the experiments in reactions containing native Luc (4 °C) or denatured Luc (43 °C) in the presence or absence of ATP. We found that the presence of ATP did not seem to affect the interaction between VCP and Luc under either condition (Figure 6b, lanes 2 vs 3 and lanes 5 vs 6). Therefore, the aggregation-prevention activity in D1 may be attributed to its innate capability of binding to non-native proteins.

A Reduced Intracellular VCP Level Results in an Increase of Luc-GFP Aggregation in Vivo, and the Increase Is Enhanced in Response to Heat Shock. To further characterize the in vitro aggregation-prevention activity of VCP, we first

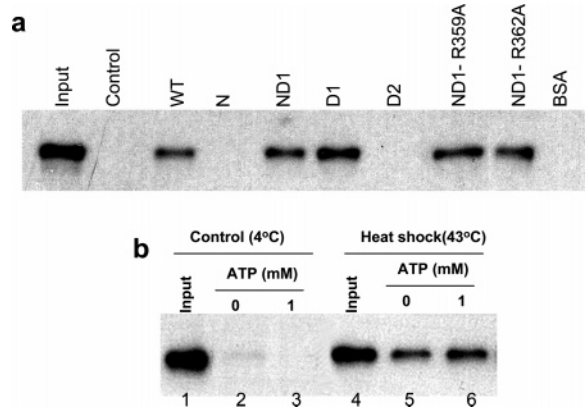

FIGURE 6: The VCP D1 domain binds to non-native Luc. Luc (100 nM) was mixed with (a) the indicated His-tagged VCP variants (1 μM each) or with (b) His-tagged D1 in the presence of 0 or 1 mM ATP at the indicated temperature. The mixtures were incubated for 10 min and then centrifuged. The soluble fractions were mixed with NTA−agarose beads to precipitate His-VCP variants. The VCP-bound Luc, which coprecipitated with VCP, was then detected by SDS−PAGE and Western blotting with Luc-specific antibody.

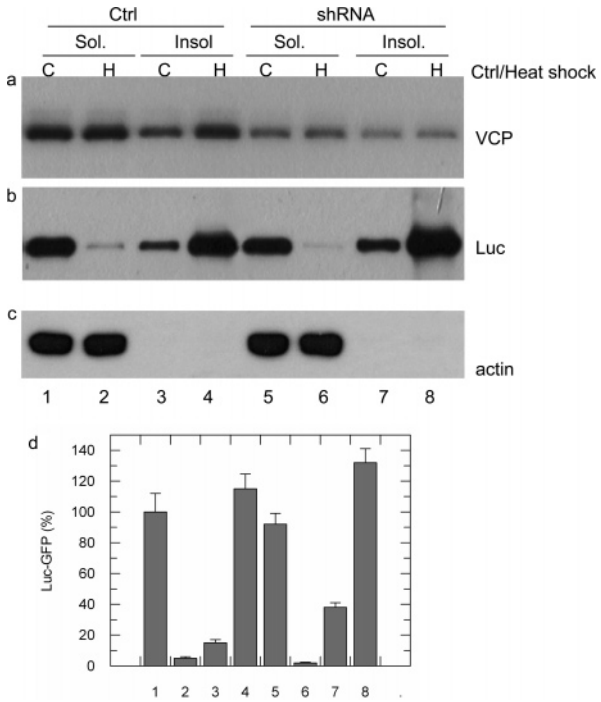

FIGURE 7: Reduction of VCP results in an increase of Luc-GFP aggregation in vivo. HEK293 cells stably expressing Luc-GFP were transfected with control vector (Ctrl) or VCP-specific shRNA vector (shRNA) for 5 days and then treated without (C) or with (H) heat shock at 43 °C for 1 h. Cells were lyzed, and the lysates were centrifuged. The supernatant (Sol) and pellet (Insol) fractions of the cell lysates were analyzed by SDS−PAGE and Western blotting using antibodies against (a) VCP, (b) Luc, and (c) β-actin. (d) The band intensity of Luc-GFP (b) was measured using Gel-Pro image analysis software and presented as the percentage of Luc-GFP. The Luc-GFP level in the control cytosolic fraction at 37 °C is set as 100%. The data presented were the average of three experiments.

examined whether reduced expression of cellular VCP enhances the extent of protein aggregation in cells. We used RNA interference-mediated gene disruption to decrease the level of VCP in the cells that express Luc-GFP and then analyzed the aggregation of Luc inside the cells. We transfected VCP short hairpin RNA (shRNA) or its empty vector (Ctrl) into HEK293 cells that stably express Luc-GFP

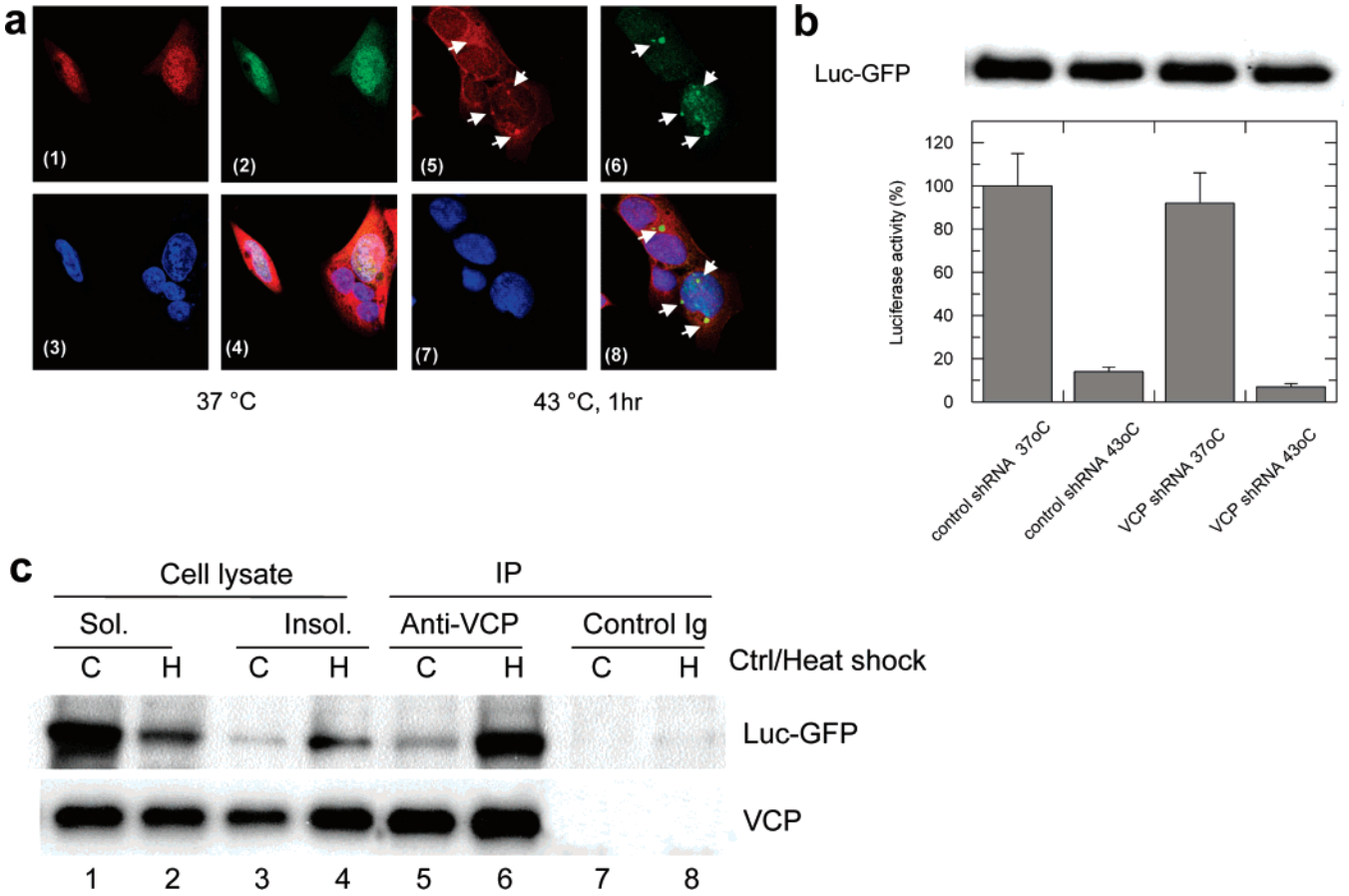

FIGURE 8: VCP proteins colocalize and associate with denatured Luc-GFP. (a) HEK293 cells stably expressing Luc-GFP fusion protein were cultured in Lab-Tek chambers and treated without (panels 1−4) or with (panels 5−8) heat (43 °C, 1 h). Cells were fixed, reacted with VCP-specific antibodies, and followed by a TRITC-labeled secondary antibody (panels 1 and 5). Hoechst staining was performed to localize the nuclei (panels 3 and 7). The processed cells were then imaged for the localization of VCP (panels 1 and 5), Luc-GFP (panels 2 and 6), and nuclei (panels 3 and 7). Panels 4 and 8 are merged images of panels 1 with 2 and panels 5 with 6, respectively. The foci containing both VCP and Luc-GFP are indicated by arrows. (b) Luc-GFP-expressing HEK293 cells transfected with VCP shRNA or its control vector were treated with or without heat, and the cell lysates were extracted and used to measure the enzyme activity of Luc. The data presented represent the average of three experiments. The Luc-GFP in the total cell lysate was detected using Western blotting with anti-Luc antibody. (c) Luc-GFP-expressing cells were treated with (H) or without (C) 43 °C heat shock and lysed. The cell lysates were separated into soluble and insoluble fractions, and a portion of the soluble lysate was immunoprecipitated with anti-VCP antibody or a mouse Ig antibody as negative control. The separated soluble fraction (lanes 1 and 2), the insoluble fraction (lanes 3 and 4) of cell lysates, and the precipitated VCP complexes (lanes 5−8) were then analyzed by SDS−PAGE and Western blotting using Luc-specific or VCP-specific antibody.

for 5 days and found that shRNA specifically reduced the level of VCP (Figure 7a, lanes 5 vs 1 and lanes 7 vs 3) but not that of actin (Figure 7c, lanes 5 vs 1 and lanes 7 vs 3). On the basis of the notion that VCP acts as a molecular chaperone that protects proteins from denaturation and aggregation, we expected to find more protein aggregates in shRNA-treated cells. Indeed, more Luc-GFP is present in the insoluble (Insol) fraction (Figure 7b,d, lane or bar 7 vs 3) and less Luc-GFP in the soluble (Sol) fraction (lane or bar 5 vs 1). Furthermore, we examined if heat stress further enhances the aggregation in shRNA-treated cells. Strikingly, when these cells were subjected to a 43 °C heat shock (H) for 1 h, more aggregates (Figure 7b,d, lane or bar 8 vs 4) and less soluble Luc-GFP (lane or bar 6 vs 2) were detected in shRNA-treated cells than in control cells. Together, these results show that a reduced level of VCP resulted in an increase of protein aggregation in vivo, and this increase was further enhanced by heat treatment.

VCP Colocalizes and Associates with Non-Native Luc in Cells. To study whether VCP interacts with thermally denatured proteins in vivo, HEK293 cells expressing thermolabile Luc-GFP were stressed at 43 °C for 1 h and then analyzed by confocal microscopy (Figure 8a). Because Luc, but not GFP, is thermolabile, heat shock will only denature Luc. Thus, after heat treatment, the fusion proteins composed of native GFP and denatured Luc will aggregate and appear as green fluorescent foci (*24*). The results show that heat shock induced the formation of prominent Luc-GFP foci (green) (Figure 8a, compare panels 2 and 6) and that VCP molecules (red) colocalized with these foci (Figure 8a, panels 5, 6, and 8). These foci marked the presence of insoluble aggregates of Luc, in which Luc was enzymatically inactive in HEK293 transfected with VCP shRNA or its control vector (Figure 8b). To further test whether VCP interacted with the non-native Luc in cells (Figure 8c), we lysed the heat-shocked (H) and the control (C) cells and separated cell lysates to soluble (Sol) and insoluble (Insol) fractions (lanes 1−4). We then immunoprecipitated VCP from the soluble fraction (lanes 5−8) and determined if Luc-GFP is present (upper panel) in VCP-containing (lower panel) complexes. The result shows that Luc-GFP is coprecipitated, thus associated with VCP in the soluble fraction, and that the association was more pronounced in heat-shocked cells (upper panel, lanes 6 vs 5). Overall, these experiments

indicate that VCP molecules colocalized with the foci containing non-native Luc-GFP (Figure 8a) and physically associated with non-native Luc in the same complex (Figure 8c).

DISCUSSION

In this report, we demonstrated that VCP has chaperone activity in suppressing protein aggregation after thermal or chemical treatment. Our in vitro studies showed that this aggregation-prevention activity was carried out mainly by the D1 domain and did not require ATPase activity or a hexameric structure of VCP. Since D1 is a highly conserved AAA domain, these findings possibly also apply to other AAA family proteins. Consistent with the in vitro results, in vivo analyses also demonstrated that a lower level of VCP correlated with a higher degree of protein aggregation in cells and that the aggregation was further increased by heat shock challenge.

Our results suggest that VCP, being an abundant protein in cells (*2*), may respond to thermal stress by binding to and maintaining the stressed non-native proteins in a soluble condition. It is conceivable that VCP may even participate in dissolving or eliminating the protein aggregates. Interestingly, we previously reported that the optimal temperature for the ATPase activity of VCP is 55 °C (*23*), a temperature significantly higher than the physiological condition. This relatively heat-resistant enzyme activity suggests that VCP may apply its ATPase activity to dissolve or to unfold/refold non-native proteins. However, our preliminary study (not shown) showed that VCP cannot perform this function alone. Therefore, it is possible that VCP cooperates with other cochaperones to fold/unfold, and possibly also to reactivate, the non-native proteins. This is supported by the finding that the Cdc48p (yeast VCP homologue) can work with Hsp40/Hsc70 to rescue inactivated proteins (*28*).

This aggregation-prevention activity may be used to rationalize other biological functions of VCP. In mediating protein degradation through the ubiquitin−proteasome pathway, VCP may separate the ubiquitinated protein from a protein complex and deliver this protein to the 26S proteasome for degradation (*17*, *29*−*31*). With this aggregation-prevention activity, VCP maintains the protein in a soluble condition and possibly further assists the unfolding of the protein. It is known that the 26S proteasome consists of a 20S proteolytic core and two 19S regulatory complexes, which attach to the two ends of the core. Because the 20S core has a narrow opening that only allows the passage of unfolded polypeptides (*32*−*35*), the substrate protein has to be unfolded before entering the 20S core. Since both the 19S complex (*36*−*38*) and VAT (an archaeal VCP homologue) (*39*) are capable of remodeling proteins, it is conceivable that VCP may cooperate with the 19S complex to facilitate unfolding and entry of the substrate into the 20S core.

The aggregation-prevention activity may also assist VCP in mediating endoplasmic reticulum-associated protein degradation (ERAD), a process responsible for the quality control of newly synthesized/assembled proteins. When an aberrant or improperly assembled protein emerges as an ER luminal protein or as an integral membrane protein, it is extracted back to the cytosolic side of ER. In the meantime, the protein is polyubiquitinated and targeted to the 26S proteasome for degradation. Due to genetic alterations or environmental changes, aberrant proteins often have surface-exposed hydrophobic patches that are highly interactive with each other. Through such interactions, these proteins bind together to form insoluble aggregates. VCP has been shown to recognize such ERAD substrates in either a ubiquitin-dependent or a ubiquitin-independent manner (*19*). In the ubiquitin-dependent process, VCP binds the ubiquitinated protein through its N domain (*18*) and, with the help of cofactors Npl4 and Ufd1, extracts the protein out of the ER membrane. In the ubiquitin-independent pathway, VCP may directly bind to the substrates via the D1 domain of VCP and the hydrophobic patches on the surface of substrate proteins.

It has been shown that a hexameric form of the VCP complex is required for the ATPase activity and for mediating ubiquitin−proteasome degradation (*13*, *25*). However, we unexpectedly found that such a structure is not required for the aggregation-prevention activity of VCP. We generated VCP mutants that are unable to form hexamers and showed that the monomeric form of D1 is sufficient to prevent protein aggregation (Figures 2 and 3). To further support this finding, we demonstrated that the wild-type ND1, which is a hexamer, and two ND1 mutants, which are not hexamers, are all capable of suppressing protein aggregation (Figure 4). These results led to a conclusion that monomeric VCP is sufficient to prevent protein aggregation. In other words, the aggregation-prevention activity of VCP is independent of its conformation, consistent with our finding that this chaperone activity is insensitive to the conformational changes induced by ATP binding or ATP hydrolysis (Figure 5).

The ready association between VCP and non-native proteins suggests a link between VCP and the diseases involving protein aggregation, such as neurodegenerative diseases and inclusion body myopathy. It has been demonstrated that VCP and its homologue, Ter94, interact with protein aggregates containing polyglutamine (PolyQ) (*40*, *41*), a hallmark of Huntington's disease. VCP also colocalizes with ubiquitin-containing inclusion bodies in the cerebral cortex of neuronal degenerative disorders (*42*). Furthermore, it has recently been shown that a fatal disease, *i*nclusion *b*ody *m*yopathy with *P*aget *d*isease and *f*rontotemporal *d*ementia (IBMPFD), results from specific VCP mutations (*43*). Remarkably, the mutant VCP proteins, primarily containing abnormal N domains, colocalize with the insoluble inclusion bodies in the muscle fibers of IBMPFD patients (*43*). It may be presumed that in the presence of non-native proteins VCP readily binds to these proteins through its D1 domain and colocalizes with the aggregates. However, because the N domain is responsible for binding to both ubiquitinated proteins and cofactors, mutations in the N domain may compromise the ability of VCP to mediate the degradation of ubiquitinated proteins. Consequently, when non-native proteins accumulate to an overwhelmingly high level, the aggregates associate with one another to form large insoluble inclusion bodies. Although the specific role of VCP colocalized with protein aggregates is not clear, based on the finding that the Cdc48 (yeast homologue of VCP)/Hsc70/Hsp40 complex can reactivate denatured Luc (*28*), VCP may have a similar function. Further studies on the chaperone activity of VCP would shed light on the molecular mecha-

nisms involved in the pathogenesis of aggregate-containing diseases.

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